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EXPERIMENTS ON THE PHYSIOLOGY OF INDIGO-YIELDING GLUCOSIDES

BY

F. R. PARNELL, B.A

Government Economic Botanist, Madras

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PREFACE.

THE following investigations were carried out whilst I was Botanist to the Bihar Planters' Association at Sirsiah Research Station. When this station was closed down in May, 1913, I hoped that I might be able to continue this work but this is found now to be very improbable. Since definite information on certain points has been obtained, the publication of the results may be of value in view of the fact that very little is known of the biology of indican in particular and glucosides in general.

I wish to take this opportunity of thanking Mr. Bergtheil, late Director of the Sirsiah Research Station, for the help and advice that he gave me throughout the work. I wish to thank also Mr. Harrison, Government Agricultural Chemist, for allowing me to finish off several experiments in his laboratory at Coimbatore.

COIMBATORE,

8th March, 1915.

F. R. PARNELL.

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EXPERIMENTS ON THE PHYSIOLOGY OF INDIGO-YIELDING GLUCOSIDES.

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F. R. PARNELL, B.A.,

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INDIGO-YIELDING GLUCOSIDES.

Occurrence.

AN indigo-yielding glucoside occurs in a number of plants representing widely separated natural orders and including trees, woody shrubs, small herbaceous plants and epiphytic orchids. As far as can be seen these plants do not possess in common any special character from which one can judge the biological significance of the glucoside.

Localization, macroscopic.

The localization of the glucoside in the plant is found to vary considerably in different species. Leake[1] examined a number of species in detail and summarized previous work, mainly of Molisch and Beijerinck, on this subject.

In *Polygonum tinctorium*, Ait., the glucoside is confined to the leaf lamina, Leake,[1] Molisch.[2]

In *Indigofera spp.* it is practically confined to the leaf and youngest parts of the stem though a small amount, varying in the different species, occurs in some of the reproductive organs, Leake.[1]

In *Isatis tinctoria*, L., Molisch[2] and Beijerinck[3] have shown it to occur in parts of the young roots in addition to the aerial vegetative organs.

In *Phajus spp.* and *Calanthe spp.* Molisch[2] finds it in the flower and roots in addition to the aerial vegetative organs.

Wrightia tinctoria, Br., is described by Roxburgh[4] as containing indigo in the leaf. In the course of the present work it was found also in the seed in considerable quantity, in the young roots throughout and in the outer cortex of the old roots. It is absent from the laticiferous vessels.

[1] Leake, H. M. *Annals of Botany*, Vol. XIX, No. LXXIV, April, 1905, p. 297.
[2] Molisch, H. *Sitzb. der Kais. Akad. d. Wiss. in Wien.*, Bd. CII, Abt. 1, 1893, p. 269.
[3] Beijerinck, M. W. *Proc. K. Akad. Wetensch. Amsterdam*, Bd. II, 1900, p. 495.
[4] Roxburgh, W. *Flora indica*, 1874, p. 585.

Wrightia tomentosa, R. & S., was found to contain none in the aerial organs but considerable quantities in the seed and roots as in *Wrightia tinctoria*.

Thus the localization of the glucoside in the plant is not suggestive of any special function common to all the plants in which it occurs; on the other hand, the important differences shown rather raise doubts as to the existence of a common function.

Localization, microscopic.

With regard to the localization inside the cell Molisch[1] considers that some special relation exists between the indican and chloroplasts and seeks to prove this by showing that, when the leaf is treated with the vapour of alcohol or ammonia, indigotin is deposited in the cell in close contact with the chloroplasts. Leake [2] shows that this is not the case when an acid persulphate fixing solution is used. Leake's solution is capable of depositing indigotin wherever it comes into contact with indican. In Molisch's method partial asphyxiation allows the indican and enzyme to interact with production of indoxyl and glucose, the former then being oxidized to indigotin either spontaneously by oxygen in solution in the cell-sap or through the agency of an enzyme. On examination it was found that an oxidase or peroxidase, or both, occurs in the glucoside-bearing parts of all the plants examined, *viz.*:—*I. arrecta*, *I. sumatrana*, *W. tinctoria*, *W. tomentosa*, *Crotalaria incana* and *Phajus sp.* It is obvious that if oxidation is brought about by this enzyme the indigotin will be deposited in close contact with the enzyme irrespective of where the original indican was localized. Even if no enzyme is concerned in the oxidation, the localization of the glucoside splitting enzyme and any possible variation in oxygen concentration in different parts of the cell would largely control the point of deposition of indigotin. Molisch's results therefore cannot be accepted as demonstrating any connection between the glucoside and chloroplasts.

Nature of Glucoside.

Schunck [3] was the first to show that the indigo-producing substance of *Isatis tinctoria* and *Polygonum tinctorium* is of the nature of a glucoside, which he named indican, capable of yielding indigotin and a sugar on hydrolysis.

Hazewinkel [4] showed that indican obtained from *Indigofera leptostachya* (=*I. arrecta*, Hochst.) is a compound of indoxyl and dextrose. This was confirmed by Perkin and Bloxam[5] who prepared large quantities of pure

[1] Molisch, H. *Ber. d. Deutsch. Bot. Gesell.* Bd. XVII, 1899, p. 228.
[2] Leake, H. M. *Annals of Botany*, Vol. XIX, No. LXXIV, April—1905, p. 297.
[3] Schunck, E. *Phil. Mag.* (4) Vol. X, 1855, p. 74, and Vol. XV, 1858, p. 127.
[4] Hazewinkel, J. J. *Proc. K. Akad. Wetensch. Amsterdam*, Bd. II, 1900, p. 512.
[5] Perkin, A. G., and Bloxam, W. P. *Jl. Chem. Soc. Trans.* Vol. XCI, 1907, p. 1715.

indican and made a careful study of its properties. They also showed that the glucoside of *I. sumatrana*, Gaert., is identical with that from *I. arrecta*.

Beijerinck [1] showed that the glucoside of *Isatis tinctoria* differs from indican in that it is decomposed by even feebly alkaline solutions whereas indican is stable in concentrated alkaline solutions.

In the course of the present work it was noticed that the glucoside of *Wrightia tinctoria* was hydrolised by acid more slowly than was indican from *I. arrecta* under similar conditions. A rough experiment designed to prove this quantitatively was carried out and the results are given in Table I. In each case to 250 c.c. of plant extract at 31°C was added 3 c.c. of strong hydrochloric acid and 3 c.c. of 10 per cent. ammonium persulphate solution After definite times the indigo precipitated was filtered off and estimated with standard permanganate solution.

TABLE I.

Acid Hydrolysis of Glucosides of I. arrecta *and* W. tinctoria.

TIME	INDIGOTIN PRODUCED = PERMANGANATE C.C.		PERCENT HYDROLISED	
	Indigo	Wrightia	Indigo	Wrightia
½ hour	3·85	3·15	67·5	36·2
do.	3·85	lost	67·5	...
1 hour	4·3	3·75	75·5	43·1
do.	4·3	3·85	75·5	44·3
4½ hours	5·7	8·7	100	100
do.	5·7	8·65	100	100

A better idea of the comparative rates of hydrolysis would have been obtained if the plant extracts had been more nearly of equal content, yet the results undoubtedly show that the *Indigofera* glucoside is more readily hydrolised by acid than is that of *Wrightia*.

A more careful experiment for comparing the action of the respective enzymes was carried out with the result shown in Table II. Here the extracts were made approximately of equal content after a preliminary estimation. In each case the enzyme powder was made by extracting the fresh leaf with alcohol of about 90 per cent. till yellowish, drying, grinding, and passing through a fine gauze sieve. It may be noted here that *Wrightia* enzyme will stand much less alcohol treatment than that of *Indigofera*, and it is necessary to get the whole extraction done in about one hour for the powder to be very active. *I. sumatrana* was used in this experiment.

[1] Beijerinck, M. W. *Proc. K. Akad. Wetensch. Amsterdam*, Bd. III, 1900, p. 101.

In each case 400 c.c. of plant extract was used, to this was added five drops of chloroform, 0·5 gm. *Indigofera* enzyme or 0·75 gm. *Wrightia* enzyme and the whole put on a shaking machine for the time stated. To stop the action 10 c.c. of strong ammonia was added and the whole shaken with air for some time to oxidize any indoxyl present. After boiling and filtering the remaining glucoside was estimated by the indirubin method.

TABLE II.

Interaction of Enzymes and Glucosides of I. sumatrana *and* W. tinctoria.

No.	Enzyme	Time	Per cent. hydrolised		Remarks
			Indigo Extract	Wrightia Extract	
1	Indigo	4 hrs.	83·0	34·1	Shaken together.
2	do.	do.	74·6	37·4	
3	do.	16 hrs.	100·0	34·1	
4	do.	do.	100·0	29·0	
5	Wrightia	4 hrs.	33·9	86·9	do.
6	do.	do.	38·1	83·6	
7	do.	16 hrs.	67·0	100·0	
8	do.	do.	72·0	100·0	

N. B.—Relative concentrations of extract: *Indigofera* 118, *Wrightia* 107.

It will be seen from the Table that *Indigofera* enzyme acts much more strongly on *Indigofera* extract than on *Wrightia* and *vice versâ*. There is not the least doubt that both the glucosides and enzymes, although very similar, are not identical.

Physiological Considerations.

Wrightia Seed and Seedlings.

Both *W. tinctoria* and *W. tomentosa*, as noted above, contain an indigo-producing glucoside in the seed. The following experiments were designed to show what effect germination and growth would have on the quantity of glucoside present.

The seeds are large and easy to handle, *W. tinctoria* being about the size and shape of an oat grain, *W. tomentosa* being rather smaller. An even batch of good sound seed was picked out and sown in well washed nitrogen-free sand in porcelain pots. These were watered throughout with nitrogen-free distilled water and lots were taken for analysis at intervals throughout germination and growth.

It was found impossible to get full germination so that the actual weight of seed giving each lot of plants could not be determined. For this reason the results are expressed in terms of numbers of seedlings. Equal numbers do not represent absolutely equal weights of seed and to estimate the experimental error thus involved weighments were made of several random lots of seed from each batch, in number the same as the lots of plants analysed. These are given at the foot of each table.

In all cases the glucoside was estimated by extraction with boiling water[1] and precipitation of indirubin by boiling with hydrochloric acid and isatin in an atmosphere of carbon dioxide. In Tables III and V the indirubin was measured tintometrically in alcoholic solution ; in Table IV it was weighed.

TABLE III.

Wrightia tinctoria *Seed germinated and grown on distilled water.*

No.	Number seedlings	Age in days	Indirubin per 16 plants	Average relative amounts	REMARKS
1 a	16	2	0·0097	1	Seed germinating, radicle $\frac{1}{4}''$—$\frac{1}{2}''$ long.
b	do.	do.	0·0093		
2 a	do.	4	0·0123	1·30	Radicle with root hairs, cotyledons about to open.
3 a	do.	7	0·0143	1·48	Cotyledons well open.
b	do.	do.	0·0139		
4 a	do.	11	0·0161	1·70	Plumule elongated.
5 a	do.	15	0·0175	1·87	1st. pr. real leaves.
b	do.	do.	0·0180		
6 a	do.	25	0·0233	2·55	2nd. do.
b	do.	do.	0·0252		
7 a	do.	38	0·0269	2·95	3rd. do.
b	do.	do.	0·0292		
8 a	16	53	0·0260	2·70	4th. do. Slightly yellowish.
b	8	do.	0·0252		
9 a	10	77	0·0177	1·74	do. very yellow, nitrogen starved.
b	do.	do.	0·0153		
10 a	do.	99	0·0248	2·50	Nitrate solution added after No. 9 analysed and plants healthy looking again.
b	do.	do.	0·0228		

Seed weight, 16=0·782, 0·905, 0·833, 0·750, Average 0·817.

[1] Very careful extraction is necessary to ensure removal of all the glucoside from the seed. The procedure was to boil 3—4 mins., filter through fine linen, grind in mortar, boil and filter again squeezing the pulp in linen, boil and filter once more. The milky extract was cleared by twice washing with ether and then boiling with a trace of ammonia and filtering through paper.

Table IV.

Wrightia tinctoria *Seed germinated and grown on distilled water.*

No.	Number seedlings	Age in days	Weight when analysed	Indirubin per 100	Average relative amounts	Remarks
A 1 a	100	*Nil.*	4·35	0·063	1	Dry seed.
b	do.	do.	4·05	0·061		
,, 2 a	do.	4	8·23	0·0685	1·19	Germinated, radicle ½″.
b	do.	do.	8·65	0·079		
,, 3 a	do.	8	10·65	0·079	1·29	Radicle 1″ with root hairs.
b	do.	do.	10·63	0·0806		
,, 4 a	112	12	30·30	0·093	1·51	Cotyledons well open.
b	do.	do.	32·70	0·095		
B 1 a	100	*Nil.*	4·41	0·071	1	Dry seed.
b	do.	do.	4·18	0·074		
,, 2 a	102	22	37·00	0·094	1·27	1st. pr. real leaves.
b	do.	do.	40·60	0·0905		
,, 3 a	77	31	42·00	0·184	2·56	3rd. do.
b	do.	do.	42·50	0·187		

Seed weight A 650=27·62, Average 100=4·25.

B 100=4·39, 4·64, 4·51, 4·65, Average=4·55.

N.B.—A and B represent seed from different sources.

Table V.

Wrightia tomentosa *Seed germinated and grown on distilled water.*

No.	Number seedlings	Age in days	Indirubin per 16 plants	Average relative amounts	Remarks
1 a	16	*Nil.*	0·00406	1	Dry seed.
b	do.	do.	0·00438		
2 a	do.	2	0·00450	1·02	Germinating, radicle ½″ long.
b	do.	do.	0·00410		
3 a	do.	10	0·00370	0·92	Cotyledons well open.
b	do.	do.	0·00405		
4 a	do.	23	0·00483	1·10	1st. pr. real leaves.
b	do.	do.	0·00447		
5 a	do.	35	0·00439	1·13	2nd. do.
b	do.	do.	0·00517		
6 a	do.	47	0·00392	0·93	3rd. do.

Seed weight, 16=0·381, 0·387, 0·414, 0·382, Average=0·391.

It will be seen from Table III that in *W. tinctoria* the amount of glucoside increases as the seedling develops until a maximum is reached in No. 7 at 38 days. After this the seedlings stop growing, begin to get yellow and show obvious signs of nitrogen starvation and the amount decreases. Nitrate solution was supplied to the seedlings at this stage and they rapidly recovered and the glucoside content increased. Table IV confirms these results on a larger scale, so far as the gradual increase of glucoside is concerned, but was not carried far enough to show the ultimate decrease. It appears that in the ordinary course of germination the glucoside of the seed does not act as a nitrogenous reserve for use in building up new tissue but actually increases at the expense of other nitrogenous matter. When, however, nitrogen starvation begins to be felt the glucoside begins to disappear, apparently being drawn on as a source of nitrogen. It is obviously not very readily available for this purpose, however, since Nos. 9 (*a*) and (*b*) of Table III were almost on the point of death from lack of nitrogen and yet contained a good deal of glucoside. It may be noted here that the glucoside nitrogen represents only slightly more than 1 per cent. of the total nitrogen of the seed.

Table V shows the very different behaviour of *W. tomentosa* under similar conditions. It is evident that the amount of glucoside remains fairly constant for the period shown. There is no large increase comparable with that of *W. tinctoria*. This is no doubt connected with the fact that in *W. tomentosa* the glucoside is confined to the root system whereas in *W. tinctoria* it is present also in the leaf and young stem. The meaning of these differences is not clear on the supposition that the function of the glucoside is the same for both species.

Owing to lack of seed the experiment could not be repeated on a larger scale and stages showing nitrogen starvation could not be included.

Consumption of Glucoside by Developing Cuttings.

Polygonum tinctorium and *Strobilanthes flaccidifolius* were chosen as indigo-yielding plants which very readily strike root when parts of the shoot are placed in water. It was possible, by making duplicate lots of cuttings, analysing one immediately and the other after growth for some time on nitrogen-free water, to detect any consumption of glucoside taking place under these conditions. Cuttings of various sorts were tried in this manner, *e.g.*, growing points with a few leaves, axillary shoots with node of origin with and without subtending leaf, single leaves with node of origin and axillary bud, etc. In all cases a number of similar cuttings was made and divided into two lots *a* and *b*

by taking pairs as nearly alike as possible ; *a* was analysed directly, *b* struck and analysed after growing for some time on nitrogen-free distilled water. The cuttings rooted freely and showed considerable increase in weight.

Tables VI and VII give the results obtained and show in every case a reduction in the amount of glucoside after growth.

TABLE VI.

Polygonum tinctorium *Cuttings*.

No.	No. of cuttings	Original weight	Days on water	Final weight	Per cent wt. increase	Indigotin percentage	Per cent. diffce.	Remarks
1 a	39	7·30	...	.....	...	0·256	—59	Axillary shoots with node of origin.
b	37	7·00	22	12·65	80·9	0·104		
2 a	36	8·15	...	...	...	0·324	—77	do.
b	35	7·74	20	13·67	76·4	0·075		
3 a	25	7·65	..	.....	...	0·266	—0·7	do.
b	25	7·21	22	12·0	66·4	0·264		
4 a	22	10·20	...	.....	...	0·310	—78	do.
b	22	10·60	22	18·4	73·8	0·069		
5 a	38	11·22	...	.....	...	0·459	—14	Same batch.
b	28	8·22	10	12·05	47	0·395		
6 a	36	15·10	...	.....	...	0·307	—13	
b	34	15·40	10	20·67	34	0·267		
7 a	50	10·87	...	.....	...	0·336	—23·5	Same batch.
b	47	9·90	24	16·2	64	0·257		
8 a	30	10·82	...	... ...	...	0·362	—27	
b	27	9·30	24	15·4	66	0·264		
9 a	18	10·80	...	.....	..	0·270	— 9·3	
b	17	9·90	18	15·6	58	0·245		

N.B.—1-4 by indigotin method,
5-9 by indirubin method,
both calculated as indigotin % of original cuttings.

TABLE VII.

Strobilanthes flaccidifolius *Cuttings.*

No.		No. of cuttings	Original weight	Days on water	Final weight	Per cent wt. increase	Indigotin per cent.	Per cent. diffce.	Remarks
1	a	12	20·4	...	...	...	0·890	−6·2	Axillary shoot with half node.
	b	12	19·5	6	22·9	17·5	0·835		
2	a	26	32·7	...	...	...	0·87	−5·7	Young axilly. shoot with subtg. leaf.
	b	25	30·6	30	38·5	25·8	0·82		
3	a	7	6·0	...	...	...	1·26	−17·5	Younger do.
	b	7	6·1	30	7·95	30·4	1·04		
4	a	6	6·3	...	...	...	1·42	−34·5	Stem apex with few young leaves.
		6	7·4	58	11·8	59·5	0·93		

N.B.—Indirubin method, calculated as indigotin % of original cuttings.

There is very considerable variation in the amount of reduction shown by different lots. In Table VI Nos. 1—4 were similar cuttings receiving the same treatment and giving a fairly uniform increase in weight; with the exception of No. 3, which appears to have gone wrong in some way, the glucoside reduction is fairly uniform. Nos. 5 and 6 are similarly uniform; of Nos. 7—9, No. 9, which is rather low, was composed of larger cuttings with a lower initial content and showed less increase in weight owing to its shorter period of growth.

In Table VII the loss of glucoside varies with the increase in weight and initial content. Material was not available for determining the relative importance of the latter factor in its relation to the reduction of glucoside nor to see whether initial total nitrogen content was of importance in this respect.

It is quite certain from the above results that cuttings containing the glucoside make use of it during their development when nitrogen is not supplied from outside. Analyses of the water on which the cuttings were grown showed no trace of glucoside so that there was no loss by diffusion through the stalks or roots.

Relation between Glucoside Content of Leaf and Stage of Development.

Indigofera arrecta and *Wrightia tinctoria* have been examined in this connection and in both cases the glucoside is found throughout the whole development of the leaf from its rudimentary condition at the growing point to the fully mature state and even after leaf-fall.

Table VIII shows the quantitative variation for different stages of *I. arrecta*. A and B represent two different plants; in series A the duplicates *a* and *b* were chosen from the same parts of the plant and as nearly alike as possible. It will be seen that the actual amount of indican per leaflet increases throughout with increase of size. The percentage, however, is not constant since the content and weight do not increase proportionately. Series B shows that during the very early stages of growth the percentage increases to a maximum and then falls regularly to a minimum at maturity. Series A shows only the decrease since it does not include sufficiently early stages to show the initial rise.

TABLE VIII.

Indigofera arrecta *developing Leaf.*

No.	Number of leaflets analysed	Weight 100 leaflets	Permang. N/100 c.c. per 100 leaflets	Indigotin percentage
A 1 a	121	0·70	8·9	0·88
,, b	114	0·67	8·6	0·89
2 a	117	0·91	10·9	0·83
,, b	109	0·87	10·1	0·82
3 a	116	1·35	12·6	0·65
,, b	108	1·23	12·2	0·69
4 a	115	2·11	19·5	0·64
,, b	126	2·16	19·3	0·62
B 1	393	0·26	3·1	0·84
2	273	0·36	4·5	0·89
3	278	0·50	6·6	0·94
4	245	0·71	8·0	0·80
5	209	0·84	9·0	0·76
6	191	1·12	10·9	0·67
7	210	1·54	13·6	0·62
8	211	1·89	13·7	0·51

N.B.—Indigotin precipitated, sulphonated and titrated with permanganate.

Table IX shows similar relations for *W. tinctoria* with the exception of the initial rise which possibly occurs at a stage earlier than is included. It might appear from Nos. 4 and 5 that some loss of glucoside from the leaf occurs in the last stages. There is little doubt, however, that this does not represent actual fact; No. 4 comprised leaves of a slightly later period of growth than No. 5 and they would have become distinctly larger than the latter at maturity since, for a time, as the growing season advances, the size of the mature leaf increases. Thus it is more than probable that No. 4 leaves,

at the degree of maturity represented by No. 5, would have been appreciably larger than the latter and the glucoside content would have been correspondingly greater.

In the second half of Table IX, *a* represents a lot obtained by taking for analysis one leaf from each of a number of pairs of just mature leaves, *b* comprising the opposite leaves which were left on the plant for one month and then analysed. In each case the *b's*, at the time of analysis, were just beginning to shed, several leaves having actually fallen and the others falling at a touch. It is obvious that the glucoside content is unaltered by the aging of a mature leaf, moreover there is no removal before leaf-fall.

TABLE IX.

Wrightia tinctoria *developing Leaf.*

No.	Number of leaves analysed	Weight 100 leaves	Indigotin per 100 leaves	Indigotin percentage
1	231	4·9	0·0374	0·76
2	100	16·4	0·109	0·66
3	30	67·5	0·263	0·39
4	14	158	0·521	0·33
5	16	173	0·388	0·22
I a	17	172	0·371	0·21
b	16	178	0·377	0·21
II a	17	201	0·388	0·19
b	17	207	0·373	0·18

N.B.—Indirubin precipitated and calculated as indigotin content.

Table X shows exactly the same for *I. arrecta*. In this case before shedding the leaf turns yellow; *b* represents such yellow leaf shaken from the plant in the early morning, *a* represents mature green leaf from the same plant taken on the same day. The indican content is obviously the same for both, showing that no withdrawal takes place before leaf-fall. The total nitrogen content as given by Kjeldahl analysis shows that a very large proportion of other nitrogenous matter has been withdrawn. Similarly whilst the green leaf contained large quantities of starch there was none in the yellow. The fact that the indican remains intact, whilst all the starch and a large proportion of the total nitrogenous matter are removed before shedding, suggests that indican is of little importance as a nutrient substance.

Since this was written Howard and Howard have stated (*Second Report on the Improvement of Indigo in Bihar*, pages 4 and 5) that indican acts as a reserve which is utilized by the plant at flowering and times of starvation. In view of the results here published the author is unable to accept these conclusions. [F. R. P.]

TABLE X.

Indigofera arrecta *falling Leaf.*

No.	Leaf	Indigotin per cent.	Equal to Nitrogen per cent.	Total Nitrogen per cent.
1 a	Green	0·45	.....	.....
b	Yellow	0·48	.. ...	.....
2 a	Green	0·44	0·047	0·97
b	Yellow	0·47	0·050	0·39
3 a	Green	0·48	0 051	0·97
b	Yellow	0·45	0·048	0·33

N. B.—Indigotin precipitated, sulphonated and titrated with permanganate.

In this connection it may be remarked that the enzymic activity is less in the yellowed leaf than in the green. It was noticed that when yellow and green leaf of equal indican content were placed to steep in water under similar conditions there was considerably less action in the case of the yellow than the green.

For a quantitative estimation of the differences some enzymically active leaf powder was made from yellow and green leaf respectively of the same plant by extraction with alcohol, under similar conditions, till colourless, drying and powdering. Equal weights, 0·4 gm., of these powders were added to 250 c.c. aliquots of plant extract at 35° C and allowed to act for 25 mins. during which time they were well shaken. After addition of ammonia and thorough shaking and boiling they were filtered and the remaining indican estimated together with a control aliquot of the plant extract. It was found that of the original indican 16·4 per cent. had been hydrolised by the green leaf enzyme powder as against 11·2 per cent. by the yellow. Thus a considerable loss of activity is shown after yellowing. The reason for this is uncertain though it is possible that part of the enzyme is removed along with the other nitrogenous material.

Connection between Light and Glucoside Production.

That indican production is not dependent on light was shown very easily by covering the stump of a cut down plant of *Indigofera arrecta* with a light-tight kerosene tin. Shoots arose from the stump and after two or three weeks these were analysed. They were typical etiolated shoots, devoid of chlorophyll, with very small leaves and long slender succulent stem. Indican was found

in all parts. Their indican content was compared with that of normal shoots of the same age, a number of whole shoots of each being cut from the stumps and analysed.

Normal	...	...	0·71 per cent. indigotin.
Etiolated	...	...	0·28 do. do.

Strict comparison is impossible owing to the different nature of the two—thus the very succulent nature of the etiolated shoots no doubt accounts partly for the low content. It is obvious that indican in considerable quantity can be produced in the dark. Since an old stump without leaves contains no indican, it could not be a matter of translocation of already existing indican.

Molisch[1] obtained the same result with etiolated shoots of *Indigofera anil*, *Marsdenia tinctoria* and *Isatis tinctoria* but was not certain that the glucoside was not translocated from normal leafy shoots which were present on the same plant in each case. On the other hand, he found that *Isatis tinctoria* seedlings produce no indican when raised in the dark and seedlings containing indican lose the whole of it when put into darkness for two or three weeks. He gives no account of the state of the seedlings after several weeks of darkness and it is impossible, for this reason, to draw definite conclusions from his results. If the seedlings dropped nearly all their leaves, which commonly happens with plants put in darkness, the indican contained in them would be lost also. The fact that they contained no indican at the end of the time would then mean that the leaves produced in darkness under those conditions formed no indican. There would be no evidence that already existing indican had been used up.

This would be in accord with the fact that seedlings raised in the dark produce no indican. On the other hand, he shows that indican is produced in etiolated shoots arising from a large plant of *Isatis*. This seeming anomaly would admit of the simple explanation that in a small seedling growing in darkness a very serious deficit of carbohydrates is bound to occur, whereas a larger plant or stump will contain sufficient carbohydrate, stored in its tissues, to carry on for a long time in darkness. Since the glucoside is directly dependent on some carbohydrate for its construction, it is more than probable that simple lack of carbohydrate is responsible for its non-production in small seedlings grown or raised in darkness.

Molisch[2] also gives results showing the effect on the indican content of keeping plants of *Indigofera sp.* in the dark for 24 hours. Batches of six

1 Molisch, H. *Sitzb. der Kais. Akad. d. Wiss. in Wien.* Bd. CVII, Abt. 1, 1898, p. 747.
2 *Loc. cit.*

plants were analysed after remaining in the open and being enclosed for 24 hours in a light-tight covering respectively. In each case the darkened plants showed a lower indican content than the normal plants left in the open, the difference varying from 6·8—47 per cent. of the normal content.

The following results obtained in this line of work differ very considerably from those of Molisch. The turgidity of the leaflet varies considerably according to the atmospheric conditions at different times during the day. This alone produces variation in the indican content when the latter is calculated as percentage of fresh weight. For this reason the results have been given also as indigotin per 100 leaflets. The error due to variation in size of the leaflet is made comparatively small by the large number taken.

Table XI gives the indican content at sunrise and sunset respectively of indigo leaves. Nos. 1 and 2 represent single plants of *I. arrecta;* No. 3 represents a batch of twelve plants of *I. sumatrana* from each of which four leaves were picked for each of the four lots included. Normal mature leaves were taken in each case and the leaflets only were analysed by the indirubin method.

TABLE XI.

Indigofera spp., *Indican Content after night and day.*

No.	Time of analysis	Number of leaflets	Weight	Weight of 100 leaflets	Indigotin per cent.	Indigotin per 100 leaflets
1 a	Sunrise	273	7·81	2·86	0·491	0·0141
b	Sunset	266	7·02	2·64	0·509	0·0134
2 a	Sunrise	315	6·86	2·18	0·373	0·00813
b	Sunset	324	6·10	1·88	0·422	0·00795
3 a	Sunrise	436	23·28	5·34	0·591	0·0315)
a′	do.	428	22·04	5·15	0·608	0·0313)
4 b	Sunset	435	22·44	5·16	0·612	0·0314)
b′	do.	448	23·07	5·15	0·614	0·0316)

It is obvious from the figures that there is no appreciable difference in indican content between the two times.

Table XII gives a comparison between the indican content of three batches of *I. sumatrana* taken after *twelve hours daylight* and *thirty-six hours darkness* respectively. Those marked *a* were analysed at sunset after about twelve hours of daylight, those marked *b*, the same plants thirty-six hours later, at sunrise, after being two nights in the open and in darkness under a tent during the intervening day. As before, mature leaves were taken and the leaflets only analysed by the indirubin method.

Table XII.

I. sumatrana *Indican Content.*

(a)—after twelve hours daylight.

(b)—after thirty-six hours darkness.

No.	Number of leaflets	Weight	Weight per 100 leaflets	Indigotin per cent.	Indigotin per 100 leaflets	Average
1 a	464	16·63	3·59	0·533	0·0191	0·0193
a'	450	16·26	3·61	0·542	0·0196	
b	428	14·14	3·36	0·563	0·0186	0·0182
b'	442	14·10	3·19	0·558	0·0178	
2 a	549	19·13	3·49	0·580	0·0202	0·0200
a'	529	19·06	3·60	0·551	0·0199	
b	502	16·48	3·28	0·589	0·0193	0·0196
b	533	18·57	3·49	0·572	0·0199	
3 a	284	11·24	3·96	0·629	0·0249	0·0242
a'	281	11·23	4·00	0·590	0·0236	
b	264	11·84	4·49	0·536	0·0241	0·0249
b	260	11·42	4·40	0·585	0·0257	

The figures prove definitely that there is no large alteration in indican content after thirty-six hours of darkness. It is probable that the small variation shown is due to experimental error in sampling and analysis, the whole nature of the experiment making it very difficult to get exact results.

It follows from these results that either indican remains unaltered in the mature leaf, taking no part in normal metabolism, or, if it takes part in metabolism, its rates of production and removal are approximately equal irrespective of whether the plant is in light or darkness.

It is not easy to see why Molisch's results showed a large reduction in indican content in the leaf after twenty-four hours in darkness. He neglected the variation due to differences in turgidity—differences that might amount to a very considerable proportion if not specially guarded against. His plants were enclosed for twenty-four hours in a covering of which he gives no description. It is quite common, when a plant is enclosed with an airtight covering, for the leaves to turn blackish and many of the young shoots to die even after twelve hours under certain conditions. Possibly he was actually measuring the effect on indican content of a pathological condition set up by the conditions of his experiment. It may be noted that the plants of Table XII

above were perfectly healthy looking after their spell of darkness since a period of only twelve hours was spent under a covering and that was large and admitted of ventilation.

Function of Glucoside.

Two suggestions have been put forward of the function of indigo-yielding glucosides, both tentatively and without definite supporting evidence.

Molisch,[1] as a result of his work to which reference has already been made, considers that some definite connection exists between indican production and carbon dioxide assimilation and suggests that indican may represent the first step in the synthesis of proteids from carbohydrates and inorganic nitrogen.

The formation of the glucoside is obviously dependent on the presence of a carbohydrate, but that there is no immediate connection with carbon dioxide assimilation follows from the fact that indican is produced in the etiolated shoots arising from an indigo stump kept in darkness. Moreover the occurrence of the glucoside in the roots only of *Wrightia tomentosa* points to the same conclusion.

With regard to its function in metabolism as the first nitrogenous organic product in the synthesis of proteids it is very unlikely that it is confined to this rôle. Indigo seed, which contains no indican, if supplied with nitrogen-free water, produces seedlings containing indican. This was not carried out under absolutely sterile conditions but no trace of nodule formation could be seen. As shown above, Tables III and IV, the amount of glucoside contained in the seed of *Wrightia tinctoria* increases considerably on germination and growth on nitrogen-free water. It is probable that the glucoside in these cases is produced at the expense of higher organic compounds by catabolic processes.

That the glucoside can be used as a nitrogenous reserve appears evident from the results given in Tables III, VI, and VII which show the reduction in glucoside content produced by the nitrogen starvation of seedlings of *W. tinctoria*, and cuttings of *Polygonum tinctorium* and *Strobilanthes flaccidifolius* respectively.

Whether this is a normal function is open to doubt. If this be the case it appears unlikely that so large an amount of glucoside would remain in seedlings on the point of death from nitrogen starvation—No. 9, Table III. Moreover it has been shown that the whole of the glucoside is thrown away at leaf-fall whereas a large proportion of other nitrogenous matter and all

[1] Molisch, H. *Sitzb. der Kais. Akad. d. Wiss. in Wien.* Bd. CVII, Abt. 1, 1898, p. 747.

the starch is removed from the leaf before it falls. This does not point to the importance of the glucoside as a reserve material.

It seems unlikely that a compound functioning as a reserve, or as a step in normal metabolism, should remain so remarkably uniform in concentration as indican is found to be. Its maximum percentage concentration occurs whilst the leaf is very small. As the leaf develops the actual content of indican increases and reaches a maximum at about maturity. The rate of increase, however, does not keep pace with the growth of the leaf, thus resulting in a gradual decrease in the percentage content. After once attaining a maximum, at the maturity of the leaf, the amount of indican remains unaltered, day and night, throughout its life and even at leaf-fall no alteration can be detected.

It is fairly certain that the seasonal variations in the percentage content of mature leaf of a plant are due to variations in the amount of indican produced during the development of different flushes of leaf. Thus it is common to find that the leaf produced during a rapid flush after rain contains a lower percentage of indican than the already existing older leaf that was produced more slowly.

This is in accordance with the well known fact that rank growth tends to low indigo content and slow growth to high content. Another example of this is seen in the usual effect of heavy nitrogenous manuring of indigo: the amount of green leaf is increased considerably but its percentage content is very much reduced.

Walther[1] suggests that indican is a *prochromogen* and that indoxyl functions as a respiratory chromogen in accordance with Palladin's theory of respiration. He shows that the other requirements of the theory, *viz.*, reducing and oxidizing enzymes, are both present, so that the suggestion is possible but remains to be proved.

It seems that considerable further knowledge on the whole subject is necessary before any conclusions can be drawn as to the function of the glucoside. There is considerable doubt as to whether the function is the same in different species and it is quite certain that the actual glucoside is not the same in all.

Summary.

The following is a summary of the conclusions arrived at as a result of the above investigations :—

1. An indigo-yielding glucoside is present in the root and seed of both *Wrightia tinctoria*, Br. and *W. tomentosa* R. and S. It is absent from the leaves of the latter.

1 Walther, Oscar. *Ber. d. Deutsch. Bot. Gesell.* Bd XXVII, 1909, p. 106.

2. The glucoside and its enzyme in *W. tinctoria* are distinct from those of *Indigofera arrecta* and *I. sumatrana* although *Wrightia* enzyme has some action on *Indigofera* glucoside and *vice versâ*.

3. *W. tinctoria* seed, when germinated and grown without nitrogen, increases in glucoside content till it becomes about trebled at about forty days. As nitrogen starvation begins to show the amount decreases but is still considerable when the seedlings are on the point of death.

4. *W. tomentosa* seedlings show no appreciable increase of glucoside under the same conditions.

5. When cuttings of *Polygonum tinctorium* and *Strobilanthes flaccidifolius* are grown without nitrogen part of the glucoside disappears, presumably being used up as a nitrogenous reserve.

6. In *W. tinctoria* and *I. arrecta* the maximum percentage content occurs at a very early stage in the development of the leaf. The actual amount in any leaf, however, increases during growth to a maximum at maturity and remains constant till leaf-fall when the whole is present in the fallen leaf.

7. Indican is produced in the dark by etiolated shoots of *I. arrecta*.

8. There is no variation in indican content between night and day in *I. arrecta* and *I. sumatrana*. Moreover no marked effect is produced by keeping plants of *I. sumatrana* in the dark for thirty-six hours.

9. In the light of present knowledge no definite function can be assigned to indigo-yielding glucosides in general or to the glucoside of any special species.

COIMBATORE,

March 1915.